On the Biological and Chemical Reactivity of Carbamylated Glucagon[1] (36252)

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Some proteins are easily carbamylated by carbamyl phosphate (CP) while others are not (1, 2). There is a great deal of variability in affinity and sensitivity of proteins to modification by CP and by other related reagents. Some proteins are carbamylated preferentially on the lysine residue, while in others, the amino acid terminal is more susceptible. This is also the case for protein carbamylation with cyanate (main decomposition product of CP at alkaline pH), although pH and other conditions do favor preferential carbamylation of lysine versus that of the terminal amino acid (3, 4).

It has been shown recently that glucagon can be acylated and that it loses activity entirely after acetylation (5). By contrast, the biological activity of beef insulin, a polypeptide containing only one lysine, is not decreased by acetylation (6), but this hormone is inactivated by carbamylation (7). It was, therefore, of interest to test whether or not glucagon would be susceptible to carbamylation and to study the effect of carbamylation on its biological activity. Moreover, since glucagon possesses one lysine in addition to the *N*-terminal histidine, it was important to determine at what position would the carbamylation occur. This paper clearly indicates that the *N*-terminal histidine of glucagon is essential for biological activity as measured by the adipokinetic and hyperglycemic effects in the duck.

Materials and Methods. Except for the initial studies which were done with commercial glucagon (Lilly), crystalline glucagon (Lot No. 258-234B-167-1), kindly supplied by Lilly Laboratories, was used. CP and urease (Type VI, 83,000 units/g) were obtained from Sigma Chemical Company. KCNO was obtained from Fisher Chemical Company. Fluorodinitrobenzene (FDNB) was purchased from Eastman Organic. Dinitrophenylamino acids were purchased from Mann Laboratories. $K^{14}CNO$ was purchased from Amersham/Searle. All other reagents were best commercial grade available.

Carbamylation. Glucagon (30 mg) in 1.5 ml were mixed with 1.5 ml of 0.2 *M* CP or 1.5 ml of 0.2 *M* KCNO. A control contained only glucagon in water. The tubes were incubated at 37° for 90 min with continuous stirring. Aliquots (0.1 ml) were then taken and incubated for 20 min with 25 μl of urease (0.25 mg); and the carbamyl protein was measured (2). Two drops of glacial acetic acid were added to the remainder of each tube. Samples (1 ml) were lyophilized and used for biological testing. Similar conditions were used with $K^{14}CNO$ [$\sim$1 mg KCNO, (50 μCi) was mixed with 200 μmoles of KCNO].

The method for determining which amino acid side chain has been substituted involved the use of amino acid analysis and dinitrophenylation.

Amino acid analysis. Analyses were carried out on acid hydrolyzed samples by standard techniques with a Beckman analyzer using an accelerated system.

Dinitrophenylation. Dinitrophenylation was carried out essentially as described by Levy (8) at 40° using a Radiometer autotitrator. The solutions of carbamylated and of

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TABLE I. Comparison of Effects of Glucagon and Glucagon Carbamyl~P on Plasma Free Fatty Acids and Blood Sugar in Ducks (means ± SE).

No. of ducks	Injection	Dose (μg/kg)	Time after injection (min) 0	5	15	30	60
			Free fatty acids (meq/liter)				
6	Glucagon	3.5	1.62 ±0.07	2.54[a] ±0.15	2.17[a] ±0.13	1.78 ±0.08	1.54 ±0.02
6	Glucagon carbamyl~P	3.5	1.71 ±0.11	1.72 ±0.09	1.72 ±0.09	1.64 ±0.06	1.61 ±0.06
6	Glucagon	7.0	1.34 ±0.11	2.82[a] ±0.05	2.19[a] ±0.08	1.77[b] ±0.17	1.53 ±0.08
6	Glucagon carbamyl~P	7.0	1.21 ±0.11	1.22 ±0.10	1.24 ±0.10	1.21 ±0.09	1.23 ±0.08
9	Glucagon	100	1.55 ±0.07	3.45[a] ±0.15	3.40[a] ±0.20	2.76[a] ±0.17	2.30[a] ±0.12
9	Glucagon carbamyl~P	100	1.60 ±0.10	2.43[a] ±0.18	2.15[a] ±0.16	1.92[b] ±0.14	1.69 ±0.12
			Blood sugar (mg/100 ml)				
6	Glucagon	3.5	132 ±6.2	167[a] ±7.3	188[a] ±12.2	155[a] ±4.4	150[a] ±4.4
6	Glucagon carbamyl~P	3.5	145 ±4.4	152[a] ±3.8	160[a] ±5.8	166[b] ±9.8	163 ±5.4
6	Glucagon	7.0	137 ±3.7	173[a] ±4.6	204[a] ±7.7	153 ±11.3	141 ±9.3
6	Glucagon carbamyl~P	7.0	140 ±6.1	150[a] ±7.5	159[a] ±7.4	161[a] ±6.3	162[a] ±4.8
9	Glucagon	100	127 ±5.7	156[a] ±6.1	195[a] ±9.9	202[a] ±14.9	189[a] ±14.8
9	Glucagon carbamyl~P	100	126 ±4.9	150[a] ±5.9	166[a] ±7.2	148 ±8.3	138[a] ±4.2

[a] Significantly higher than corresponding preinjection value: $p < .01$; [b] $.01 < p < .05$.

noncarbamylated glucagon (1.0 ml) were mixed with 1.0 ml of 0.3 *M* KCl, pH was adjusted to ~7 with 10 *M* KOH, then to 8.0 with 0.1 *M* KOH, the volume was made to 3 ml with H_2O, and 0.2 ml of FDNB was added. After 90 min (to ensure complete dinitrophenylation), the mixtures were extracted three times with 5 vol of water-saturated diethyl ether. The dinitrophenyl-protein (DNP-protein) was precipitated with 3 drops of concentrated HCl and washed successively with 0.01 *M* HCl, acetone, and ether. After removal of ether, each precipitate was hydrolyzed for 16 hr at 110° in 2 ml of 6 *N* glass distilled HCl in sealed evacuated Pyrex tubes. After removal of HCl on a rotary evaporator, the hydrolysates were suspended in 1 ml of 0.1 *N* HCl and extracted 3 times with 5 vol of ether followed by 3 extractions with 5 vol of ethyl acetate. These extracts were evaporated to dryness separately and resuspended in 0.5 ml of ether (Fraction A) and ethyl acetate (Fraction B), respectively. The extracted fractions (acid-water soluble, Fraction C) were also analyzed.

Aliquots of Fractions B and C were chromatographed on silica G plates (Eastman Kodak) using *n*-butanol–33% ammonia (80:20) and *n*-propyl alcohol–34% ammonia solution (70:30) in one-dimension chromatography. Aliquots of Fraction A were chromatographed utilizing toluene–pyridine–ethy-

lenechlorohydrin–0.8 *N* ammonia (100:30:60:60) for first dimension and chloroform–benzyl alcohol–glacial acetic acid (70:30:30) for second dimension. These conditions were selected from those recommended by Brenner *et al.* (9).

Determination of biological activity. Adult male mallard ducks were the animals used in this study. These birds are one of the avian species shown to respond to the administration of glucagon with marked elevations of plasma free fatty acid (FFA) and blood sugar (BS) concentration (10, 11). The animals were kept and fed as previously described (10, 12) and were used after 18 hr of fasting. Glucagon and its carbamyl derivatives, dissolved in glycine buffer (0.1 *M*, pH 9.5) in 0.9% NaCl, were injected into a wing vein. The solutions were prepared daily just before the injection. Blood samples were taken from the wing veins into centrifuge tubes containing heparin and kept in a bath of ice water. Plasma FFA were determined by a modification of Dole's method, as previously described (10, 12). BS was determined with *o*-toluidine as described by Hyvärinen and Nikkilä (13).

The data were analyzed by standard statistical methods. Unless stated otherwise, the levels of significance of the differences reported were calculated by Student's *t* test for paired variates.

Results. A comparison of commercial glucagon with crystalline glucagon revealed no differences in glycogenolytic activity and in effect on FFA mobilization in the intact duck. The effects of various doses of glucagon and of glucagon carbamylated with CP (glucagon carbamyl~P) on plasma FFA and BS are presented in Table I. The doses of 3.5 and 7.0 μg/kg of glucagon caused significant elevations of plasma FFA concentration, but no effect was detected with the corresponding doses of glucagon carbamyl~P. Glucagon carbamyl~P, however, caused significant elevation of plasma FFA when given at the dose of 100 μg/kg. The mean FFA elevation 5 min after injecting this dose was 0.83 ± 0.15 mEq/liter; whereas the elevation caused by the same dose of crystalline glucagon was 1.90 ± 0.15 mEq/liter. The difference between these two mean elevations (1.07 ± 0.20) was highly significant ($p < .0002$, t test for nonpaired variates).

The two lower doses of glucagon carbamyl~P caused significant elevation of BS, but, as shown in Table I, these elevations were smaller than those produced by the corresponding doses of glucagon. Thus, the BS elevation produced by 3.5 μg/kg of glucagon, 15 min after the injection, was 56 ± 7.1 mg/100 ml; whereas, the same dose of glucagon carbamyl~P caused an elevation of 21 ± 7.1 mg/100 ml, 30 min after the injection. The difference between these two elevations (35 ± 10.0 mg/100 ml) was significant at the level of $p < .01$ (t test for nonpaired variates).

The FFA response measured by the area described by the FFA concentration curve above the preinjection level for 1 hr after the injection is closely correlated with the logarithm of the dose (μg/kg). Using the data in Table I plus those of a series of experiments in ducks with various doses of crystalline glucagon, the correlation between $\log_{10}$ of glucagon dose and effect was calculated. For 10 doses (from 2.0 to 100.0 μg/kg) in 60 birds, the correlation coefficient was $r = +0.972$. The relationship between $\log_{10}$ of the dose (x) and effect, expressed by the area under the curve of FFA concentration (y), is described by the regression equation:

$$y = 24.36\,x - 5.63$$

Using the equation and the data of Table I, it was calculated that the effect on plasma FFA of 100 μg of glucagon carbamyl~P corresponds to that of 4.6 μg/kg of crystalline glucagon.

Similar calculation was made with the blood sugar data. The correlation coefficient between dose and effect was $r = +0.966$ and the regression equation was:

$$y + 17.73\,x + 3.68.$$

The effect on BS of 100 μg/kg of carbamyl glucagon corresponded to that of 3.6 μg of crystalline glucagon.

The elevation of FFA above the preinjection level of 5 min after the injection was also correlated with the dose of glucagon for doses between 2.0 and 50 μg/kg. With the

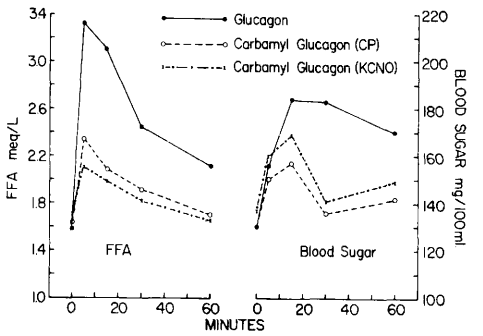

FIG. 1. Comparison of effects of crystalline glucagon, glucagon carbamyl~P, and glucagon KCNO on plasma free fatty acids and blood sugar concentration in ducks (100 μg/kg iv each, at 0 min); means of 6 ducks/group.

data of 8 groups of ducks (48 ducks) injected with the 8 doses covering that range, the correlation found was $r = +0.970$. The regression equation relating $\log_{10}$ of glucagon dose (μg/kg) (x) and FFA elevation (mEq/liter) (y) at 5 min is:

$$y = 1.04\,x + 0.18.$$

With this equation it was calculated that the effect on plasma FFA of 100 μg of glucagon carbamyl~P corresponds to that of 4.2 μg/kg of crystalline glucagon. Similar results were obtained with glucagon carbamylated with KCNO. Figure 1 shows that the effects on plasma FFA and BS of glucagon carbamyl~P and of glucagon KCNO are comparable.

As shown in Table II there is no indication of carbamylation of lysine as evidenced by dinitrophenylation or by amino acid analysis. Moreover, no homocitrulline was detected by amino acid analysis of hydrolyzed carbamylated glucagon. As outlined in Table II, the experimental evidence clearly indicates that only the NH_2-terminal histidine has been carbamylated, since no di-DNP histidine was detected in the carbamylated samples. The low chromogenicity of the carbamylated product indicates that there is no carbamylated lysine. However, the observed chromogenicity agrees with that calculated for carbamyl histidine. Since small amounts of free histidine could escape detection on thin-layer chromatography, it was of interest to quantitate the extent of carbamylation by a different method, and this was carried out by the use of ^{14}C labeled cyanate exactly under the conditions used for the experiment of Fig. 1. As shown in Table II, 77% of the glucagon was carbamylated.

Discussion. Our data show that carbamylation of the *N*-terminal histidine of glucagon results in marked loss of the hyperglycemic and adipokinetic effects of this hormone. Carbamylated glucagon retains some adipokinetic and glycogenolytic effects as shown by the results obtained with injection of a large dose (100 μg/kg) of the carbamylated preparation. The calculations reported indicate, however, that these effects are smaller than those produced by ~5.0 μg of crystalline glucagon. As shown in Table II, some 23% of glucagon has not been carbamylated; therefore, there is approximately 5 times more noncarbamylated glucagon than needed to produce the observed effects. This consideration indicates that carbamyl glucagon is devoid of adipokinetic and glycogenolytic effects *in vivo*, and suggests the possibility that the carbamyl derivative may act as an inhibitor of glucagon. In this connection it is of interest to note that the carbamylated derivatives of oxytocin exhibit no activity on the isolated uterus, but inhibit the action of the hormone (14, 15).

The fact that there was no increased carbamylation above ~75%, under somewhat more drastic conditions than those described above, may be ascribed to the intrinsic sensitivity of protein, polypeptides, and even amino acids, to carbamylation, whether or not reflecting steric hindrance (16), with either cyanate or carbamyl phosphate. Although it is too early to generalize, the examples thus far available indicate that, in some cases, there are mixed populations of carbamylated protein, for example, with hemoglobin. In some cases, once a certain amount of protein is carbamylated, it is difficult to obtain additional carbamylation. Whether this is due to aggregation or some other effect is unclear. In this regard, it should be noted that often there is a large decrease in solubility following carbamylation; this decrease in solubility may, by occlusion, prevent further carbamylation.

TABLE II. Carbamylation of Glucagon with C~P and KCNO.

	(μmoles/μmole of glucagon)		
	Control	Carbamyl phosphate	KCNO
Colorimetric method[a]			
Carbamyl histidine	—	0.84 ± 0.07	0.96 ± 0.05
$K^{14}CNO$ incorporation	—	—	0.77 ± 0.02
Dinitrophenylation[b]			
di-DNP-histidine	1.0 ± 0.08	<0.05	<0.05
εDNP-lysine	0.96 ± 0.10	0.93 ± 0.10	0.95 ± 0.10
Amino acid analysis[c]			
Lysine	1.35	1.22	
Histidine	0.93	0.75	

[a] These calculations were based upon the chromogenicity of carbamyl histidine using the method previously described (1). The chromogenicity of carbamyl histidine is 13% of that observed with homocitrulline. The increase in chromogenicity of treated samples of glucagon over that found in control samples was considered to be due to carbamyl histidine formation. All samples were incubated with an excess urease prior to assay.

[b] Calculations were based upon the DNP derivative eluted from chromatographic plates after separation.

[c] The bulk of the carbamylated histidine would be decomposed during the 16 hr hydrolysis in 6 *N* HCl. For compactness of presentation, the additional amino acids are not listed since there were no differences.

It is of interest that, as shown by others, glucagon may be acetylated on both the NH_2-terminal histidine and the ε-amino group of lysine with retention of gross physicochemical properties including immunological reactivity (5). These workers attempted to differentiate the importance of the *N*-amino terminal residue and the ε-amino group of lysine for activity by the use of differential reagents. Acetylation results in modification of both the *N*-terminal histidine and the ε-amino group of lysine. Under their conditions, there was complete loss of biological activity. On the other hand, pyridoxal phosphate, reacts only with ε-amino group of lysine; and, under these conditions, glucagon is reported to lose its glycogenolytic properties (5). The present experiment illustrates that *both* the glycogenolytic and the adipokinetic effects are prevented when glucagon is carbamylated and *that only the N-terminal amino acid needs to be modified.* Furthermore, only the alpha-amino group of histidine is expected to be carbamylated, because carbamylimidazol is extremely unstable (17) and no traces of this compound can be left under the conditions of our preparation.

Our results are in agreement with those of previous reports showing that removal of the *N*-terminal histidine of glucagon results in loss of its glycogenolytic effect *in vivo*, and of its lipolytic effect *in vitro* (18, 19). A recent report (20), appeared since the completion of the present studies, shows that the amino terminal histidine residue of glucagon is essential for biological activity, as measured *in vitro* by activation of the adenylate cyclase system of liver plasma membranes or fat-cell "ghosts," but is not primarily responsible for the binding of glucagon to its receptor. These results, together with those reported here, give support to the view that activation of adenyl cyclase is essential for the development of the adipokinetic and glycogenolytic effects of glucagon *in vivo*. The possibility that carbamylated glucagon may act as a competitive inhibitor of the binding of glucagon to its receptor is raised by these studies.

As noted, acetylation of insulin does not decrease its biological activity (6); whereas its carbamylation results in gross physical changes and in a variety of products, as well as in loss of biological activity (7). One could thus selectively acetylate glucagon

without affecting insulin and this may be of practical interest.

Finally, it should be remembered that, as illustrated before and further exemplified here, cyanate and carbamyl phosphate behave in a similar manner insofar as carbamylating agents. This is of interest since carbamyl phospate may act via cyanate in carbamylation reactions. As recently shown under certain conditions, for example decomposition of carbamyl phosphate, via the specific acetyl phosphatase, cyanate is not an intermediate. Finally, the physiological carbamylating agent is carbamyl phosphate, since it is produced in large quantity by mammals as well as by other animals while cyanate is not.

Summary. Glucagon was carbamylated with carbamyl phosphate and with KCNO. Only the NH_2-terminal histidine was carbamylated. Carbamylation of glucagon resulted in loss of its adipokinetic and hyperglycemic effects in the intact duck, for the two forms of carbamylation used. These results indicate that the *N*-terminal histidine of glucagon is essential for the biological activity of this hormone.

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